Chemopreventive efficacy zingerone (4-[4-hydroxy-3-methylphenyl] butan-2-one) in experimental colon carcinogenesis in Wistar rats

Majid Ahmad Ganaie | Abdulaziz Al Saeedan | Hassan Madhkali | Basit Lateef Jan | Tanvir Khatlani | Ishfaq Ahmad Sheikh | Muneeb U. Rehman | Khalida Wani

1Department of Pharmacology, College of Pharmacy, Prince Sattan Bin Abdulaziz University, Al-Kharj, Saudi Arabia
2Department of Clinical Pharmacy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
3Stem Cells and Regenerative Medicine Department, King Abdullah International Medical Research Center, King Abdulaziz Medical City, Ministry of National Guard Health Affairs, Riyadh, Saudi Arabia
4King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia
5Department of Biochemistry, Govt. Medical College (GMC-Srinagar), Srinagar J&K, India
6Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas

Correspondence
Dr Majid Ahmad Ganaie, Department of Pharmacology, College of Pharmacy, Prince Sattan Bin Abdulaziz University, P.O. Box 173, Al-Kharj, 11942, Saudi Arabia.
Email: majidsays@gmail.com

Funding Information
Deanship of Scientific Research at Prince Sattan Bin Abdulaziz University, Grant/Award Number: 2016/03/6564

Abstract
Colorectal cancer is one of the most common cancers worldwide. Development of naturally occurring inexpensive and safe alternatives can be effective in suppressing colon related proliferations. Zingerone (4-[4-hydroxy-3-methylphenyl] butan-2-one), a polyphenolic alkanone of ginger, has massive pharmacological properties and thus can be used as promising candidate against various ailments. In the current study, we aimed at demonstrating the protective effect of zingerone against experimental colon carcinogenesis and elucidating its possible mechanism by studying inflammatory and Nrf-2 signaling cascade. Four groups of animals (I-IV) were made with six animals each. Group I (control) was given normal saline orally. Group II was given 1,2-dimethylhydrazine (DMH) at the dose rate of 20 mg/kg body weight. Group III and IV were treated with DMH at the dose rate of 20 mg/kg body weight and also received oral treatment of zingerone at a dose rate of 50 and 100 mg/kg body weight, respectively, for first 5 weeks and animals were euthanized after 16 weeks. Our results reveal that DMH treated rats exhibited elevated ROS and MDA levels, increased activity of cytochrome P450 2E1 and serum marker enzyme carcinoembryonic antigen (CEA), increased no of aberrant crypts of foci (ACF), and elevated expression of inflammatory and proliferative proteins. Nrf-2 was downregulated by DMH treatment. Treatment with zingerone to DMH treated rats, resulted in alterations in the activity of the cytochrome P450 2E1 and CEA. In addition, immunostaining of NF-κB-p65, COX-2, iNOS, and PCNA, Ki-67 was suppressed by zingerone. Furthermore, zingerone administration also attenuated the level of IL-6 and TNF-α and it also helps in preserving mucous layer. Thus, zingerone could be considered as a good chemopreventive agent in experimental model of colon carcinogenesis. Further studies are required to study other pathways involved in colon carcinogenesis and their modulation by zingerone.

KEYWORDS
chemoprevention, colon cancer, hyperproliferation, inflammation, natural product, zingerone

1 INTRODUCTION
Cancer is the leading cause of death in developed and developing nations. In under developed countries, it is the second most cause of death. Colon cancer is the third most frequent malignancy and fourth most common cause of mortality in both the genders throughout the world. It arises due to varied alterations (genetic as well as epigenetic) in the colonic epithelium. Epidemiological studies have shown that there exists an interaction between diet and the prevalence of colorectal cancer which can be promoted by a diet rich in fat and meat.

Experimental colon carcinogenesis is induced by 1,2-dimethylhydrazine (DMH) in rodents mostly in distal part of the colon. DMH induced pathological and molecular events are similar to those observed in human colon cancer. This model finds extensive application in...
studying the molecular events, prevention strategies of colon cancer. Bulk of DMH is metabolized in liver to azoxymethane (AOM), which in presence of cytochrome P4502E1 is further metabolized to methyloxymethanol (MAM).

In liver, glucuronic acid forms a conjugate wherefrom it gains its entry into colon through bile as MAM glucuronide. Later in the colon, it is acted upon by gut microbial enzymes especially β-glucuronidase and is deconjugated to produce MAM. Formation of oxygen-derived free radicals and intermediary products of oxygen, for example, hydrogen peroxides are thought to be instigated by active metabolites of nearly all carcinogens. Colon cancer is many times a pathological repercussion of sustained oxidative stress and inflammation. Reportedly DMH induces oxidative DNA damage in the colon and liver by its capability to produce free radicals.

Because of oxidative insults, aberrant crypt foci (ACFs) are observed in colon and rectum of patients and are hence recognized as one of the earliest identifiable mucosal abnormality of the colon. With regards to the similar genotypic and morphological characteristics of ACF in animal model and human pathologies, detection of ACF is considered as an early and reliable biomarker to screen and diagnose early stages of pathogenesis of colon cancer. Thus, growth and development of DMH induced ACF in animal models is used as a reliable short-term bioassay to assess' chemopreventive potential of natural products.

Nrf-2 is a key transcriptional factor involved in activation of cytoprotective genes in response to generation of reactive oxygen species (ROS). It is ubiquitously distributed in various tissues in human as well as animals. Nrf2 is reported to have a major role in maintaining the oxidative stress homeostasis by increasing expression of antioxidative and detoxifying enzymes. Nrf2 triggers activation of the ARE-mediated antioxidative responses, AREs further are crucial for maintaining cellular redox homeostasis in both stressed and non-stressed conditions. Nrf-2 very often exposed to ubiquitin mediated proteasomal breakdown by its integrant Keap1 (Kelch-like echinoderm hydridin [ECH]-associated protein 1) under dormant circumstances. Decoupling of Nrf2 from Keap1 occurs upon triggering of oxidative stimuli. Thereby it translocates into nucleus where transcription of detoxifying and antioxidants encoding genes takes place.

Evidence from recent past has revealed that Nrf2 and NF-κB are inter-related. By regulation of NF-κB transcription factor, Nrf2 plays a very important cytoprotective role by acting as anti-inflammatory, The NF-κB signaling pathway has a very critical part in controlling cell survival and cell death by stimulating expression of downstream mediators comprising TNF-α, COX-2, and i-NOS. The progression of tumor cell metastasis also involves disproportionate expression of inflammatory metabolites. Inflammation-associated events are reported to be involved in the progress of human and DMH-induced colon carcinogenesis. Considering the huge role of Nrf2 and NF-κB it is implied that targeting by natural or alternative products could enhance the Nrf2/ARE pathways while suppress the NF-κB/inflammatory pathways in colon cancer.

Ki-67 is a nonhistone nuclear protein and is reported to be over expressed in many malignancies including colon cancer. There are number of published reports which show that Ki67 was highly expressed in malignant than in normal tissues. PCNA, a 36 kDa cofactor of DNA polymerase-δ, is one of the downstream effectors of the activation of MAPK/ERK1/2 signaling and is a reliable molecular biomarker of hyperproliferation. PCNA and Ki67 are frequently studies as markers of cellular proliferation in chemopreventive studies. Expression of both these proliferative markers correlated with the progress of neoplastic disease and are used to assess the hyperproliferative events.

Development of drugs from natural sources is emerging as a key strategy to identify novel anticancer agents. Numerous naturally occurring phytochemicals have been evaluated for anticancer properties attributed to their ability to interfere with multiple pathways controlling survival of cancer cells. Ginger (Zingiber officinale, Roscoe Zingiberaeaceae) is regarded to have many health benefits in alternative medicinal therapies. Due to wide array of phytochemical constituents present in ginger, it has been documented to have many promising health benefits. Many active compounds like shogaols, gingerols, paradols, zingerone, etc are present in ginger. These bioactive metabolites are known to have very strong antioxidant activity due to the presence of unsaturated ketone moiety. Zingerone or vanillyl acetone or [4-[4-hydroxy-3-methylphenyl] butan-2-one is found in variable concentrations in ginger and is produced from shogaols and gingerol on thermal degradation or cooking by retro-aldol reaction. Zingerone belong to phenolic alkanone group, having many essential pharmacological properties like antioxidant, anti-diabetic, anticancer, anti-inflammatory, and antimicrobial activities.

The conventional therapies available for controlling malignancies are usually expensive and often associated with harsh side effects; therefore, various alternative therapies are increasingly sought by patients. Keeping in mind the pharmacological effects of zingerone we designed the present study to explore the possible beneficial role of zingerone on colon toxicity, we examined the protective actions of zingerone against inflammatory signaling and hyperproliferation using a DMH-induced rat model of colon cancer.

2 | METHODOLOGY

2.1 | Chemicals

Bovine serum albumin (BSA), EDTA, poly-γ-lysine, tris-base, tris-HCl, NADPH, Mayer’s hematoxylin, Alacin blue, toluidine blue, propidium iodide, methylene blue DMH, and zingerone were obtained from Sigma (Sigma Chemical Co., St Louis, MO). Poly-HRP plus ONE detection System (Thermo Scientific, Waltham, MA). All other chemicals used were of highest purity grade available.

2.2 | Animal study

Four- to six-week-old, male albino rats (120-150 g) of Wistar strain were obtained from Experimental Animal Care Center, College of
Pharmacy, Prince Sattam Bin Abdulaziz University. They were maintained under standard laboratory conditions of relative humidity (45%-55%), temperature (23-25°C) and light (12 hours light/12 hours dark), with free access to standard pellet diet and tap water throughout the experimental period. All experimental protocols described in the manuscript were duly approved by the Institutional Animal Care and Use Committee of Prince Sattam University and were conducted in accordance with NIH guidelines.

2.2.1 Preparation of carcinogen
DMH was dissolved in distilled water containing 1 mM EDTA to ensure the stability of the chemical just prior to use and the pH adjusted to 6.5 with 1 M NaOH solution.

2.3 Experimental design of the medium term study

Group I: Vehicle treated group. In this group, rats will receive basal diet and water ad libitum along with distilled water (vehicle of zingerone) orally and EDTA solution (vehicle of DMH) subcutaneously (Table 1).

Group II: DMH treated group. In this group, rats will be treated with DMH (dissolved in 1 mM EDTA solution pH adjusted to 6.5 with 1 mM NaOH) once a week for 5 weeks at the dose of 20 mg/kg body weight, subcutaneously in the groin.

Group III: Zingerone and DMH treated group (prevention group I). In this group, rats will be first treated with zingerone (50 mg/kg body weight) 2 weeks before the DMH treatment (as in group II) and zingerone will be continued till the termination of the experiment.

Group IV: Zingerone and DMH treated group (prevention group II). In this group, rats will be first treated with zingerone (100 mg/kg body weight) 2 weeks before the DMH treatment (as in group II) and zingerone will be continued till the termination of the experiment.

All the rats were sacrificed after 16 weeks and were evaluated for the colon toxicity and early markers of colon carcinogenesis, that is, aberrant crypt foci (ACF), ROS measurement, inflammatory, and proliferative markers were studied.

2.4 Measurement of ROS
ROS were measured based on the oxidation of 2’,7’-dichlorodihydrofluorescein diacetate to 2’,7’-dichlorofluorescein as described by Chan-Min Liua et al.36

2.5 Assay for cytochrome P4502E1
Cytochrome P4502E1 activity was measured by the method of Watt et al.37 The assay mixture contained 40 mM p-nitrophenol, 100 μg microsomal protein and 0.1 M phosphate buffer. Addition of 10 mM of NADPH helped in the initiation of reaction, the reaction mixture was then incubated at 37°C for 60 minutes. About 20% TCA was added to stop the reaction, it was then centrifuged at 1000 rpm for 5 minutes, 10 mM NaOH was added to the supernatant and the absorbance was measured at 450 nm. The values are expressed as mmol of p-nitrocatechol liberated/min/mg protein.

2.6 Measurement of MDA
The assay of lipid peroxidation (LPO) was done according to the method of Wright et al. The reaction mixture consisted of 0.58 mL phosphate buffer (0.1 M, pH 7.4), 0.2 mL PMS, 0.2 mL ascorbic acid (100 mM), and 0.02 mL ferric chloride (100 mM), in a total volume of 1 mL. This reaction mixture was then incubated at 37°C in a shaking water bath for 1 hour. The reaction was stopped by the addition of 1 mL trichloroacetic acid (10%). Following the addition of 1.0 mL thio-barbituric acid (TBA) (0.67%), all the tubes were placed in a boiling water bath for a period of 20 minutes. The tubes were shifted to an ice bath and then centrifuged at 2500g for 10 minutes. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm. The results were expressed as nmol TBA formed/h per g tissue at 37°C by using a molar extinction coefficient of 1.56 × 10^5/M per cm.38

2.7 ACF assay
ACF assay was done by the method of Bird. Colons picked up in random order were stained for 6 minutes in a 0.05% filtered solution of methylene blue. The numbers of ACF per colon were counted under light microscope at ×40 magnification.39

2.8 Alcian blue-neutral red staining for mucin analysis

The colonic sections of 4 mm were cut from formalin-fixed, paraffin-embedded tissue blocks, and mounted on poly-L-lysine coated microscopic slides. Paraffinized sections were dewaxed in xylene and rehydrated through graded series of ethanol to water. The sections were stained with 1% Alcian blue (pH 2.5) in 3% acetic acid solution for 30 minutes and then rinsed for 1 minute in 3% acetic acid solution to prevent nonspecific staining. The slides were then washed in distilled water and the sections were then counterstained with neutral red (0.5% aqueous solution) for 20 seconds, dehydrated in alcohol and mounted by using mounting media. The slides were then evaluated under the light microscope.36

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Tabular representation of experimental schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Treatment</td>
</tr>
<tr>
<td>Group I</td>
<td>Distilled water (vehicle of zingerone) orally and EDTA solution (vehicle of DMH) subcutaneously.</td>
</tr>
<tr>
<td>Group II</td>
<td>DMH (dissolved in 1 mM EDTA solution pH adjusted to 6.5 with 1 mM NaOH) once a week for 5 weeks at the dose of 20 mg/kg body weight, subcutaneously in the groin.</td>
</tr>
<tr>
<td>Group III</td>
<td>Animals will be first treated with zingerone (50 mg/kg body weight) 2 weeks before the DMH treatment (as in group II) and naringenin will be continued till the termination of the experiment.</td>
</tr>
<tr>
<td>Group IV</td>
<td>Animals will be first treated with zingerone (100 mg/kg body weight) 2 weeks before the DMH treatment (as in group II) and naringenin will be continued till the termination of the experiment.</td>
</tr>
</tbody>
</table>

All the animals were sacrificed after 16 weeks.
2.9 | Immunohistochemical staining of NF-κB-p65, COX-2, i-NOS, Nrf-2, Ki-67, and PCNA

The processed colon tissues were obtained and preserved in 10% paraformaldehyde overnight followed by dehydration in 30%, 20%, and 10% sucrose solution successively up to 3 days, and were then fixed in formaldehyde fixative until immunohistochemical staining. Then, 5-15 μm thick sections of paraffin-embedded tissues were cut using a microtome and boiled in 0.1 M citrate buffer (pH 6.0) for 5 minutes for the antigen retrieval process, and then incubated in 0.3% H2O2 in methanol followed by incubation in blocking buffer containing 0.1 M PBS, 0.04% Triton X-100, and 10% normal goat serum. Tissues were stained with antibodies against Anti-rat NF-κB-p65 rabbit antibody (dilution 1:100) Anti-rat COX-2 polyclonal antibody (dilution 1:100), antirat Nrf-2 rabbit antibody (dilution 1:100), antirat Ki-67 polyclonal antibody (dilution 1:200), and antirat PCNA polyclonal antibody (dilution 1:200) overnight at 4°C. After rinsing in the buffer, the sections were processed using a three-layer peroxidase staining kit. The peroxide complex was visualized with 3,3-diaminobenzidine. Finally, the slides were counterstained with hematoxylin for 5 seconds. The slides were then cleaned in sterile HPLC-grade water, gradually dehydrated with ethanol, cover slipped in mounting medium, and photographed under microscope.

2.10 | Assay for carcinoembryonic antigen

The activity of carcinoembryonic antigen (CEA) enzymes in serum was measured by ARCHITECT CEA enzyme chemiluminescent microparticle immunoassay test kit (Abbott, Ireland Diagnostic Division, Sligo, Ireland) as per the manufacturer’s protocol.

2.11 | Assay for tumor necrosis factor alpha

Tumor necrosis factor alpha (TNF-α) levels were determined by rat TNF-α kit (eBioscience, Inc., San Diego, CA). The method is based on enzyme-linked immunosorbent assay (ELISA). We have performed measurement of TNF-α in the colonic tissue by ELISA. Samples were prepared in phosphate buffered saline (PBS) containing protease inhibitor cocktail. Analysis was performed by Elisa Plate Reader (Multiskan EX,
Thermo Scientific, Waltham, MA) according to the manufacturer’s instruction.

2.12 | Assay for interleukin 6

Interleukin 6 (IL-6) was assayed by rat IL-6 kit (eBioscience, Inc., San Diego, CA). The method is based on ELISA. We have performed measurement of IL-6 in the colonic tissue by ELISA. Samples were prepared in PBS-containing protease inhibitor cocktail. Analysis was performed by Elisa Plate Reader (Multiskan EX, Thermo Scientific) according to the manufacturer’s instruction.

2.13 | Statistical analysis

The data from individual groups were presented as the mean ± SE of the mean (SEM). Differences between groups were analyzed using analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test and minimum criterion for statistical significance was set at $P < 0.05$ for all comparisons.

3 | RESULTS

3.1 | Effect of DMH and zingerone on phase I xenobiotic metabolizing enzymes (microsomes)

The activities of phase I enzymes were significantly increased ($***P < 0.001$) on DMH treatment. Zingerone regulates the activity of xenobiotic metabolizing enzymes of phase I, cytochrome P4502E1 in the colon mucosa of experimental and control animals. Supplementation with different doses of zingerone

**FIGURE 5** Effects of zingerone and DMH on incidence of ACF per rat colon. The rat colon showing round and elongated ACF with different crypt multiplicities. The colons were opened, stained with methylene blue and observed on a glass slide. Values are expressed as Mean SEM. Zingerone non-significantly suppressed the development of ACF in Group 3 and significantly ($#p < 0.05$) Group 4 as compared to DMH treated group (Group 2) [Color figure can be viewed at wileyonlinelibrary.com]
significantly ($#p < 0.01$) diminished the activity of cytochrome P4502E1. A more pronounced effect being observed in the rats supplemented with zingerone at 50 and 100 mg/kg body weight (Figure 1).

### 3.2 Effect of zingerone on ROS levels in colonic tissue

ROS level significantly increased ($*** p < 0.001$) in DMH treated group (group II) as compared with vehicle control (group I). The data shows that DMH treatment causes oxidative stress by enhancing reactive oxygen species production in the rats colon. Zingerone can lower the ROS levels in the colon of both Group II ($**p < 0.01$) and Group III ($# p < 0.01$; Figure 2).

### 3.3 Effect of zingerone on MDA levels

Figure 2 shows the data on the effect of zingerone on the levels of lipid peroxidation of control and experimental rats. In DMH alone treated rats (group II), the levels of were significantly ($*** p < 0.001$) increased in the colon as compared to the control (group I). On the other hand, zingerone supplementation (group III and IV)

---

**FIGURE 6** Photomicrographs depicting mucin staining. In DMH treated group (Group II), there is regional depletion of mucin in the form of mucous layer (blue in color). Treatment with zingerone decreased the depletion of the mucous layer in Group IV as compared to Group II. No effects of zingerone on mucous layer in Group III as compared to Group II. Last panel shows semi-quantitative evaluation of mucin immunostaining. Significant differences were indicated by $*** p < 0.001$ when compared with group I and $### p < 0.001$, $## p < 0.01$ when compared with group II [Color figure can be viewed at wileyonlinelibrary.com]
to DMH treated rats during the experimental period of 16 weeks restored (***p < 0.001) the levels of lipid peroxidation to near those of control (Figure 3).

3.4 | Zingerone regulates CEA

There was marked rise in level of CEA in DMH-treated group II (***p < 0.001) compared to control. Following DMH induction, zingerone treatment showed significant (##p < 0.01, ###p < 0.001) decline in CEA levels as compared to control group I (Figure 4).

3.5 | Effect of zingerone and DMH on the development of ACF

In DMH treated group (group II), the number of ACF/colon is 9.89 while supplementation with zingerone in group III (7.46)
nonsignificantly lowered down the count of ACF while in group IV it significantly lowered down the count of ACF 6.88 (Figure 5).

3.6 | Effect of naringenin and DMH on the mucin staining

In group II (DMH treated group), a local mucous layer depletion (blue colored) was observed. Treatment with zingerone mitigated the mucous layer depletion in group IV but not in group III as compared to DMH treated group. No mucous layer depletion was observed in colons of group I and group V (Figure 6).

3.7 | Effect of zingerone on Nrf-2

Control group showed normal immunohistochemical expression of Nrf2. DMH induced rats showed markedly lesser expression of Nrf2. Treatment with zingerone enhanced the Nrf2 expression levels as compared to DMH treated group (Figure 7).

3.8 | Effect of DMH and zingerone on Cyclin D1

In DMH treated group II, the cyclin-D1 levels were found to be significantly (***p < 0.001) higher as compared to group I control. Zingerone treatment significantly mitigated (##p < 0.01) the level of cyclin-D1 in both group III and IV as compared to group II (Figure 8).

3.9 | Effect of zingerone on inflammatory signaling

Immunohistochemistry patterns of NFκB, COX-2, and i-NOS protein expression in the tissues of colon showed immunoreactivity of all these inflammatory markers were increased in DMH treated rats (Figures 9–11; group II) than in control (group I). Visible reduction in expression of all these inflammatory markers NFκB, COX-2, and i-NOS was observed in the groups treated with zingerone (group III and IV) compared to DMH only treated group (group II). Also, in DMH treated group II, TNF-α and IL-6 levels were found to be significantly (***p < 0.001) higher as compared with group I control. Treatment with zingerone at both the doses markedly reduced the level of both TNF-α and IL-6 (Table 2). Hence, we propose that zingerone can inhibit inflammatory signaling by regulating the above proteins.

3.10 | Effect of naringenin and DMH on the expression of Ki-67 and PCNA

Ki-67 and PCNA are established markers of hyperproliferation. We observed that DMH treated group (group II) have more Ki-67, PCNA, immunopositive staining as indicated by brown color as compared with control group (group I). Treatment of zingerone reduced the immunostaining of both hyperproliferative markers studied PCNA and Ki-67, in group III and IV as compared with group II. For immunohistochemical analyses, brown color indicates specific immunostaining of PCNA and Ki-67 and light blue color indicates hematoxylin staining (Figures 12 and 13).

4 | DISCUSSION

In current study, we have demonstrated the therapeutic potential of zingerone in hyperproliferative and inflammatory signaling pathways and role of Nrf2 in experimental model of colon carcinogenesis.

The phase I, hemoproteins family constitutes of important enzymes involved in procarcinogen activation to carcinogens and cytochrome P450 is one among them.40 Since DMH is a procarcinogen, it instigates mutations that can trigger oncogenic transformations. It achieves so by undergoing metabolic activation by cytochrome P450 2E1 and generates electrophilic substances that form DNA adducts.41,42 Therefore, in chemically induced carcinogenesis, carcinogen activating enzyme expression is a critical component as it holds a very high proliferative potential of pushing usual cells to a cancerous phenotype.43,44 In the present study, the raised the activities of the cytochrome P450 family enzymes in colonic mucosa DMH treated rats was observed. The possible reason behind this could be the existence of DMH provoking substrate caused the activation of these enzymes. Treatment with zingerone to DMH treated rats aborted the CYP450 2E1 activities (Figure 1). Since, in xenobiotic toxicity, phase I enzymes are hypothetically crucial, their toxicity can therefore largely be minimized by suppression of phase I enzymes. Therefore, the defensive effect of zingerone in opposition to DMH induced colon carcinogenesis may be accredited to its power of curtailing phase I enzyme activity.

Recent studies report the role of ROS production including singlet oxygen, hydroperoxides, H₂O₂, and O₂⁻ in origination of DMH toxicity.26,45–47 The present study demonstrated the oxidative damage in colon led by excessive ROS production on DMH exposure. Previously published reports reveal that the antioxidant potential of β-sitosterol is responsible for its anticarcinogenic property in DMH-induced colon cancer.48 Besides supplementation with kaempferol has significantly restored the levels of antioxidant enzymes in DMH induced rat model of colon cancer.49 In agreement with these published reports, zingerone retracts DMH-induced colon-toxicity by controlling the reactive oxygen species level in the rats’ colon (Figure 2), that may be attributed to its capacity to target and prevent the generation of free radicals.
Methyl free radical generated by metabolism of DMH cause accumulation of hydroxyl radical or hydrogen peroxide that may contribute to lipid peroxidation or MDA. Lipid peroxidation is a known marker of oxidative stress in colon toxicity and that remarkable elevation in the level of MDA, a lipid peroxidation product, was observed after DMH treatment. Our results corroborated with the previous studies which revealed marked decrease in MDA levels after zingerone treatment (Figure 3).

Carcinoembryonic antigen (CEA) is an adhesion molecule found intracellularly. It is reported to be over expressed in colon malignancies; hence, it finds wide application as independent prognostic tumor marker. Studies from different research groups have reported the increased CEA levels in colorectal carcinoma. DMH, a potent carcinogen causes remarkable alterations in CEA enzymes levels by instigating reactive oxygen species induced injury to colon cells. We observed, an increased level of CEA in DMH induced rats as compared to control and this increase was ameliorated by the treatment with zingerone (Figure 4).

Morphological ACF are identified as endpoint putative lesions that are preadenomatous in nature. They are present inside the mucosa of colon in rodents and are exploited to assess the effect of

![Figure 9](https://wileyonlinelibrary.com)
modulating factors. In colon cancer, they are reviewed as outstanding identification markers of preneoplastic condition in humans as well as experimental models. ACF is known to carry the K-ras oncoproteins that aids in the abnormal cell growth and mutations in the adenomatous polyposis coli (APC) at the molecular level. Studies conducted in past reveal the depletion in number of ACF in an azoxymethane (AOM)-kindled rat colon cancer model on dietary treatment with quercetin, curcumin, silymarin, ginseng, aloin, and rutin. Hesperetin supplementation has also blocked ACF formation in colonic cancer rats. Maintaining this background, our research group has already reported the incidence of exorbitant number of total aberrant crypts in DMH-treated rats. Our observations in the current study revealed that treatment with zingerone led to the decreased number of aberrant crypts foci in both of the groups on comparison with DMH treated animals (Figure 5).

The lumen of the colon is constituted by goblet cells. They are extremely specialized epithelial cells which emit mucin. Mucins are large molecular weight chief glycoproteins of mucus which are secreted by epithelial cells of colon. Mucous overspreads the cell surfaces lining urogenital tracts and form protective layer in the
Intestinal lumen. Injury of any toxic nature can instigate mucin loss and inflammation. Recently conducted studies have explored the mucin modulatory potential of natural products in vitro and in vivo. Khan et al. revealed that glycyrrhizin add-on diets have exhibited attenuation in depletion of mucous layer. In the recent past, research team led by Sangeetha et al. has also reported increased mucin content on treatment with silibinin in DMH-induced rat model of colorectal carcinogenesis. Our results reveal decreased mucin

**TABLE 2** Effect of zingerone on serum levels of inflammatory cytokines (TNF-α and IL-6)

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/mL)</td>
<td>420.16 ± 37.7</td>
<td>1306.14 ± 62.4 ***</td>
<td>1003.62 ± 36.8 #</td>
<td>774.12 ± 44.8 ##</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>942.35 ± 40.6</td>
<td>2279.52 ± 61.5 ***</td>
<td>1543.08 ± 42.8 ##</td>
<td>1122.48 ± 31.9 ###</td>
</tr>
</tbody>
</table>

Results obtained are significantly different from group I (**p < 0.001**).
Results obtained are significantly different from group II (# p < 0.05) (## p < 0.01) and (### p < 0.001).
depletion in response to zingerone as compared to DMH which displayed notable mucin depletion (blue color; Figure 6).

The nuclear factor erythroid 2-related factor 2 (Nrf2) is identified as a redox-sensitive transcription factor that offers cytoprotection in response to oxidative stress and controls phase II antioxidant gene expression. In normal cells, Nrf2 is segregated by kelch-like ECH-associated protein 1 (Keap1) that stimulates its swift proteasome-facilitated breakdown. However, during oxidative stress, Nrf2 is stabilized by detached from Keap1, and attached to cis-elements called antioxidant response elements (AREs).\textsuperscript{15} Nrf-2 acts as a major regulator for the cellular defense against oxidative and electrophilic stresses by inducing enzymes that are involved in the detoxification and elimination of ROS, RNS, and electrophiles through conjugation and excretion.\textsuperscript{23} Studies conducted in the recent past reveal that in various human cancers, there is upregulation of Nrf2 expression. This suggests its possible involvement in different cancer stages like initiation, progression, and resistance to chemotherapy.\textsuperscript{68} Reportedly, antioxidant enzyme levels and Nrf-2 expression has been found to be limited during colon cancer development.\textsuperscript{69} We also observed steep decline in expression of Nrf-2 on DMH treatment and zingerone administration regulated the normal

**FIGURE 12** Photomicrographs depicting immunohistochemical staining of Ki-67: For immunohistochemical analyses, brown colour indicates specific immunostaining of Ki-67, and light blue colour indicates haematoxylin staining. The colonic section of DMH-treated group (Group II) has more Ki-67, immunopositive staining as indicated by brown colour as compared to control group (Group I) while treatment with zingerone in Group III and Group IV reduced Ki-67, immunostaining as compared to Group II. Lower panel shows semi-quantitative evaluation of Ki-67 immunostaining. Significant differences were indicated by ***$p < 0.001$ when compared with group I and ##$p < 0.01$ when compared with group II [Color figure can be viewed at wileyonlinelibrary.com]
level of Nrf-2 (Figure 7). It is believed that Nrf2 and NF-κB signaling pathways interact to influence the transcription of downstream target proteins like TNF-α, IL-6, etc. Also, Nrf-2 and NF-κB are main pathways regulating the intricate balance of cellular redox status and responses to inflammation and stress related triggers. The interplay between these pathways occurs through a wide range of complex molecular interactions and can depend on the type of cell and tissue.

NF-κB inside nucleus modulates the transcription of wide variety of genes including proinflammatory mediators, such as COX-2, i-NOS, some cytokines chemokines, and adhesion molecules. Also, some earlier studies report that activation of NF-κB facilitates transcriptional up-regulation of COX-2 and proinflammatory cytokines, such as TNF-α, IL-6, etc. DMH which generates lot of oxidants in colon tissue activates redox sensitive transcription factor NF-κB. Zingerone was found to inhibit DMH induced activation of NF-κB as it significantly reduced the levels of phosphorylated form of inhibitor of kappa B (IκB) and arrested the nuclear translocation of NF-κBp65 (Figure 9). Once in active state, the genes encoding TNF-α, COX-2, and i-NOS are transactivated by NF-κB. Results here suggest that an increased immunopositive staining of i-NOS and COX-2 and also
elevated level of TNF-α in DMH treated rats as compared to control animals. Treatment with zingerone significantly attenuated the immunopositive staining of COX-2 and iNOS and also normalized the TNF-α level (Figures 10 and 11; Table 2). These results suggest that zingerone has strong anti-inflammatory potential.

Uncontrolled cellular proliferation is the hallmark of process of multi stage colon carcinogenesis.73 Two such proliferative proteins which are associated with growth of colon cancer are proliferating cell nuclear antigen (PCNA) and Ki-67. PCNA is a 34 kDa nonhistamine nuclear protein, and is a specific marker of cell division. It is synthesized just before the S-phase of the cell cycle and is known to be associated with DNA polymerase.74 Strong evidences have suggested correlation between PCNA expression and degree of malignancy and vascular infiltration in colorectal carcinomas.75 Ki-67 is expressed during all cell cycle phases (G1, S, G2, and mitosis), except for the G0-phase.76 Expression of the Ki67 protein is linked with the proliferative activity of intrinsic cell populations and is hence used as a marker of tumor progression.77 We observed in our study there was steep increase in expression of these two markers after DMH treatment, however zingerone treatment inhibited hyperproliferative response. Our results were in accordance with reports of Rehman et al.26 and Khan et al.47

In brief, the results in this study define that zingerone exhibits excellent chemopreventive potential in experimentally induced colon tumorigenesis via suppression of reactive oxygen species and checking the development of precancerous lesions and hyperproliferation, regulate the inflammation mediated by Nrf-2 and NFκB. Our study also establishes that amelioration of Nrf-2 mediated inflammatory signaling and regulation of cell proliferation might be a part of fundamental mechanisms associated with inhibition of colon cancer by zingerone.

ACKNOWLEDGMENT
This project was supported by the Deanship of Scientific Research, at Prince Sattam Bin Abdulaziz University under the research project number 2016/03/6564. The funding source had no role in designing, writing and in the decision to submit the article for publication.

CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ORCID
Majid Ahmad Ganaie https://orcid.org/0000-0001-6895-5370
Muneeb U. Rehman https://orcid.org/0000-0002-9995-6576

REFERENCES
3. Humphries A, Wright NA. Colonic crypt organization and tumorigene-
5. Newell LE, Heddie JA. The potent colon carcinogen 1,2-dimethylhy-
7. Perse M, Cerar A. Morphological and molecular alterations in 1,2-dimethylhydrazine and azoxymethane induced colon carcinogene-
14. Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxi-
18. Lin W, Wu RT, Wu T, Khor TO, Wang H, Kong AN. Sulforaphane sup-
19. Li Y, Shen L, Luo H. Luteolin ameliorates dextran sulfate sodium-
induced colitis in mice possibly through activation of the Nrf2 signal-
21. Wu Y, Zhou BP. Inflammation: a driving force speeds cancer meta-
22. Suganuma M, Okabe S, Marino MW, Sakai A, Sueoka E, Fujiki H. Essential role of tumor necrosis factor alpha (TNF-alpha) in tumor pro-
23. Gonzalez-Domíquezes A, Alonso-Molero J, Fernandez-Villa T, Vilioro-
1572.
26. Rehman MU, Rahman Mir MU, Farooq A, et al. Naringenin (4,5,7-trihydroxyflavanone) suppresses the development of...


